Lactococcus lactis and *Lactobacillus sakei* as bio-protective culture to eliminate *Leuconostoc mesenteroides* spoilage and improve the shelf life and sensorial characteristics of commercial cooked bacon

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Running headline: Quality improvement of cooked bacon.

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Abstract

Cooked bacon is a typical Italian meat product. After production, cooked bacon is stored at 4 ± 2 °C. During storage, the microorganisms that survived pasteurisation can grow and produce spoilage. For the first time, we studied the cause of the deterioration in spoiled cooked bacon compared to unspoiled samples. Moreover, the use of bio-protective cultures to improve the quality of the product and eliminate the risk of spoilage was tested. The results show that *Leuconostoc mesenteroides* is responsible for spoilage and produces a greening colour of the meat, slime and various compounds that result from the fermentation of sugars and the degradation of nitrogen compounds.. Finally, *Lactococcus lactis spp. lactis* and *Lactobacillus sakei* were able to reduce the risk of *Leuconostoc mesenteroides* spoilage.

Keywords: Cooked bacon, spoilage, bio-protective cultures.

1. Introduction

The tradition of Italian charcuterie has ancient origins and is commonly traced back to the Roman period. Forty-three of the 315 typical traditional Italian meat products consist of different types of bacon. The most popular type is cooked bacon. Bacon is derived from the central part of the cover fat from the half of the carcass that goes from the sternal region to the inguinal. The meat is worked in different ways (natural, cured, smoked, or cooked) and then stretched or rolled in shape. The production process is reported in Table 1. The brine is composed of water and various ingredients, such as salt, fructose, dextrose, spices, ascorbic acid (E 316) and nitrate (E 252), depending on the recipe and the concentration of the ingredients. Sucrose may be used, too. The shelf life is approximately 90 days.

After pasteurisation, the microorganisms that survived pasteurisation can grow and spoil the product during storage. The species involved in the spoilage of cooked meats are heterogeneous. *Brochothrix thermosphacta, Enterococcus faecalis, Lactobacillus sakei, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc carnosum* and *Carnobacterium divergens* are widely known to negatively affect the flavour, texture, and colour of sliced and vacuum packed meat products (Bjorkroth et al. 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008; Audenaert et al., 2010; Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012). Moreover, the growth of heterofermentative bacteria can cause the packaging to swell or rupture due to the presence of CO_2. Bacterial activity is induced by the presence of the sugars that are added with the brine during tumbling, resting, or cooling for a long time. Lactic acid bacteria (LAB) and *B. thermosphacta*, which can come from the fresh meat, from the handling-related operations during processing or from the environment, are able to produce discoloration, greening and slimes (Bjorkroth et al., 1998; Audenaert et al., 2010, Vasilopoulos et al., 2010a,b; Comi et al., 2012). Improper cooking and sanitisation practices are frequent in craft cooked meat plants; consequently, the precautions are not effective in eliminating the handling-related post-contamination or the presence of

thermotolerant bacteria (Franz and von Holy,1996; Comi et al., 2012). Therefore, the use of various post-cooking or post-slicing technologies, such as high pressure, antimicrobial treatments, organic acids and protective cultures, for the bio-preservation of cooked meat products has been suggested (Metaxopoulos et al., 2002; Vermeiren et al., 2004).

The purpose of this study was to identify and characterise the spoilage-associated microorganisms in artisanal cooked bacon and to examine the use of bio-protective cultures to reduce the risk of *Leuconostoc mesenteroides* spoilage.

2. Materials and methods

2.1 Identification of microorganisms responsible for the spoilage

2.1.1. Samples and sampling procedures.

Two hundred cooked bacon of an artisan meat cooking plant were prepared following the traditional procedures of the plant (the ingredients and concentrations are subject to confidentiality). After cooking, the products were cut into 200 pieces (100 g each), packaged under vacuum, pasteurised at 85 °C for 15 minutes and stored at 4 ± 2 °C. The packaging film was Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a., Italy). The shelf-life of the products is 90 days, but just at 30 days, 40 (20 % of total) cooked bacon were spoiled, showing greening parts, slime and slightly inflated packages. At 30$_{th}$ day, 10 out of 40 spoiled and 10 out of 160 unspoiled cooked bacon samples were randomly collected and analysed. At 90 days 10 spoiled and 10 unspoiled were also analysed. The collected samples were subjected to visual and olfactory inspection and to microbiological and physico-chemical analyses. The volatile compounds of the slightly inflated packages were also studied. The rest of the products (150 samples) remained unspoiled till the end of the shelf-life.

2.1.2. Microbiological analysis

Ten grams of each sample was tenfold diluted in bacteriological peptone water (0.1 g/L peptone; 7

g/L NaCl) and homogenised in a Stomacher (P.B.I: International, Italy). Further decimal dilutions were made in the same solution, and the following microbiological analyses were performed in triplicate on agar plates: the total viable count (TVC) on Plate Count Agar (PCA, Oxoid, Italy) incubated at 30 °C for 48 hours (ISO 6887), LAB on de Man-Rogosa-Sharpe (MRS) agar (pH 6.2; Oxoid, Italy) at 30 °C for 48-72 h (ISO 15214) in a microaerophilic environment (gas pack anaerobic system, BBL, Becton Dickinson, USA), Clostridia on Differential Reinforced Clostridia Medium (DRCM) (VWR, USA) that was incubated at 37ºC for 24-48 h in an anaerobic jar with an anaerobic kit (gas pack anaerobic system, BBL, Becton Dickinson, USA), *Salmonella* spp. according to the ISO 6579 method, briefly: (1) a pre-enrichment in buffered peptone water for 18 h at 37 °C; (2) a selective enrichment of 0.1 mL of pre-enriched culture in 10 mL on Rappaport- Vassiliadis Soy (RVS) broth for 24 h at 42 °C and of 1 mL of pre-enriched culture in 10 mL of Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth for 24 h at 37 °C; (3) selective isolation by streaking 10 μ L of RVS and 10 μ L of MKTTn on plates of XLD agar and brilliant green agar for 18-24 h at 37 °C; Confirmation of any presumtive colony was performed by Salmonella Latex agglutination test (Oxoid, Italy); and *Listeria monocytogenes* according to the ISO 11290 method, briefly: (1) a primary selective enrichment in 225 mL of Half Fraser Broth for 24 h at 30 °C; (2) a secondary selective enrichment of 0.1 mL of primary enriched culture in 10 mL of Fraser Broth for 48 h at 37 °C; (3) a selective isolation obtained by streaking 10 μ L of the secondary enriched culture on Listeria Selective Agar according to Ottaviani and Agosti (ALOA) for 48 h at 37 °C.

Sixty (60) colonies (30 from the spoiled and 30 from the unspoiled samples) grown on MRS agar were randomly collected. Briefly, from one agar plate containing between 30 and 300 colonies of each samples, 3 colonies were isolated. The colonies were selected independently of morphology, colour or size. The isolates were streaked on MRS agar and then stored at -80 °C in MRS broth supplemented with glycerol (30% final concentration, Sigma-Aldrich, Germany). The isolates were

subjected to Gram staining and a catalase test and were then identified according to the molecular method (PCR-DGGE and sequencing) reported by Iacumin et al., (2009). In particular, the DNA was amplified with primers P1V1GC (GC-GCGGCGTGCCTAATACATGC) and P2V1 (TTCCCCACGCGTTACTCACC) (Cocolin et al., 2001; Rantsiou et al., 2005), the PCR products were run in DGGE, and the isolates were grouped according to the migration profile. Where possible, at least three isolates for each group were subjected to sequencing for identification purposes. A culture-independent approach was also used to identify the strains found in both the spoiled and unspoiled cooked bacon: 10 mL of the homogenised sample in bacteriological peptone water was centrifuged at 10000xg, and the pellet was then subjected to total DNA extraction, PCR-DGGE, cloning of the DGGE bands and sequencing, following the protocol used by Iacumin et al., (2009). The 60 colonies were then evaluated at strain level in order to choose the three strains to use as cultures for the challenge test against starter cultures. The test divided them in three main genotipic groups, also based on high, medium and low fermentation speed (data not shown). The isolates of unspoiled bacons had low fermentation speed and growth and this can explain the reduced concentration at the end of shelf-life of the products and consequently they can not spoil them.

2.1.3. Physico-chemical analysis and Total Volatile compounds determination

The potentiometric measurement of pH was made using a pH meter (Radiometer, Copenhagen, Denmark). The volatile compounds in 10 spoiled and 10 unspoiled cooked bacon samples, all packaged under vacuum, were analysed at the end of their shelf-life (90 days). The volatile compounds were determined by SPME-GC-MS on Finnigan Trace DSQ (Thermo Scientific Corporation, USA) with a Rtx-Wax capillary column (length 30 m x 0.25 mm id.; film thickness 0.25 µm; Restek Corporation, USA) according to the method reported in Chiesa et al., (2006). The volatiles compounds were then identified by comparison of their mass spectra and the retention times with those of standard compounds, or by comparison of the mass spectrum with those

of the mass spectrum Wiley library (Wiley library 10 vers.) and the self-made library. The results represent the average of all 10 samples analysed in triplicate.

2.1.4. Statistical analysis

The microbial concentrations of the spoiled and the unspoiled cooked bacon were compared using one-way analysis of variance. The means were separated by Tukey's honest significant difference test using the StatGraphics software package from Statistical Graphics (Rockville, Maryland).

2.2. Evaluation of starter cultures to eliminate microorganisms responsible for spoilage

2.2.1 Sample preparation, storage conditions and sampling methods for the trials using bio-protective starter culture

New pieces of cooked bacon were prepared and after chilling were inoculated with the starter cultures. The pasteurized phase was eliminated. In particular the pieces were divided into 3 lots of 10 pieces (50 g each). Lot 1 was directly inoculated with a suspension of *Leuc. mesenteroides* at a final concentration of 3 log CFU/g and used as a control. Lot 2 was inoculated with a mixture of *Leuconostoc mesenteroides/Lactococcus lactis* subsp. *lactis* (ratio 1/1) at a final concentration of 3 log CFU/g. Lot 3 was inoculated with *Leuc. mesenteroides/L. sakei* at a ratio of 1/1 and a final concentration of 3 log CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial suspension (about 6.3 log CFU/ml) on the bacons surface. After inoculation, the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser S.p.a., Italy) and stored at 4 ± 2 ºC for 90 days, at the end of their commercial shelf-life. At the end of storage, the presence of greening and of slime was evaluated, and the pH was measured.

L. sakei (B-2 Safe Pro® – CHR HANSEN) and *Lc. lactis* subsp. *lactis* (Rubis – CHR HANSEN) were obtained from the Italy branch supplier of Chr. Hansen, Denmark. The lyophilised cultures were resuspended in peptone water [0.1% sodium chloride and 0.7% peptone (Oxoid, England)] and left for 1 h at room temperature for complete rehydration. Appropriate dilutions were made, and 0.1

mL of each dilution was plated on MRS agar (de Man-Rogosa-Sharpe agar, pH 6.2, Oxoid, Italy) and incubated at 30 °C for 48-72 h in a microaerophilic environment (gas pack anaerobic system, BBL, Becton Dickinson, USA) to measure the concentration. Appropriate suspensions of the strains were mixed with *Leuc. mesenteroides* and directly inoculated on pieces of bacon, and the final bacterial cell concentration was approximately 3 log CFU/g.

The inocolum of *Leuc. mesenteroides* was represented by the a mix of three different strains isolated from spoiled cooked bacon and responsible for the greening color and spoilage. The three strains were chosen among the three genotipic groups. One of them had fast, one medium and one low fermentation speed. Despite different times of growth, the strains selected were able to produce the spoilage.

These microorganisms were grown on MRS agar, and a suspension was made for inoculation as described above. The inoculated samples (Lot 1,2,3) were analysed according to the microbial analysis above reported.

The bacterial population, using all the MRS plates from – 3 to – 7 dilutions was suspended in Phosphate Buffered Saline (one tablet Phosphate Buffered Saline, Sigma-Aldrich P4417-100TAB, St. Louis, USA, dissolved in 200 mL H_2O) and used for Bulk formation as previously described by Iacumin et al., (2011). One hundred µL of the bulk was used for DNA extraction and subjected to PCR-DGGE as above reported.

2.2.2 Sensory analysis

To evaluate the influence of bioprotective culture on the organoleptic characteristic of the product, a sensory analysis was performed using the triangle test methodology (ISO 4120:2004).

New pieces of cooked bacon, prepared following the traditional procedure of the plant, were divided into 3 lots. The lot A (10 pieces of 50 g each) was inoculated with a suspension of *L. sakei* and Lot B (10 pieces of 50 g each) by *Lc. lactis* subsp. *lactis*, both the strains were obtained from

the Italy branch supplier of Chr. Hansen, Denmark. The final concentration was for both 3 log CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial suspension (about 6.3 log CFU/ml) on the bacons surface. Lot C (10 pieces of 50 g each) was the control obtained with post-pasterurization and without adding bioprotective starter cultures. All the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a., Italy), and stored at 4 ± 2 ºC.

All the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a., Italy), and stored at 4 ± 2 ºC. Ten days before the end of their commercial shelf-life, all the lots were used for the triangle test.

The triangle test was used to compare the lot A and B to Lot C. Twenty non-professional assessors were presented with three products, two of which were identical. The assessors were asked to state which product they believed was the odd one out. The assessors who indicated there were two distinct samples were asked to identify the best one.

3.0 Results and discussion

3.1 Microbial analysis of spoiled and unspoiled cooked bacon

The concentration of LAB in the spoiled product was significantly different from the concentration in the unspoiled product ($p < 0.05$). In particular it was 8.7 ± 0.1 CFU/g in spoiled and 4.0 ± 0.2 CFU/g in unspoiled. Similarly, a significant difference ($p < 0.05$) was observed in the TVC concentration between the spoiled and unspoiled product. The spoiled samples presented a TVC concentration of 6.3 ± 0.3 CFU/g, whereas the unspoiled product presented a TVC concentration of 3.0 ± 0.5 CFU/g. The colonies growth on TVC were presuntively identified as LAB, by GRAM stain and catalase tests. The difference between the TVC and LAB concentration depends on the media used. Plate Count Agar used for TVC count tends to limit the growth of LAB and for this reason LAB concentration valued in MRS was higher than the one valued on PCA.

231 The data of the spoiled bacon show that LAB can develop in vacuum-packed meat products that
232 are stored at 4-8 °C and cause spoilage (Comi and Iacumin, 2012). The differences observed in the
233 levels of TVC and LAB are reflected by significant differences in the pH ($p < 0.05$). The pH values
234 of the unspoiled products were approximately 5.6 ± 0.1, and the spoiled products showed pH values
235 of approximately 5.3 ± 0.1. Moreover, a green colour, a slime and a slight inflation of the packaging
236 was observed in spoiled products. Diez et al., (2013) in a previous study demonstrated that *L.*
237 *mesenteroides* grew rapidly and influenced the drop in pH and produced milky exudates (slime) in
238 vacuum-packaged morcilla de Burgos a traditional blood sausages from Spain, during cold storage.
239 In leuconostocs the pathways/genes associated with many types of spoilage reactions, such as meat
240 greening, gas and slime production, and pH changing are well studied and known (Jääskeläinen et
241 al., 2013).
242 At 90 days (end of the shelf-life) the LAB concentration of the unspoiled bacons did not change. It
243 was at level of 4.1 ± 0.5 CFU/g and quite similar than the one at 30 days ($p < 0.05$). At 90 days
244 also the LAB concentration of the spoiled bacons did not deeply change, being at level of 8.5 ± 0.5
245 CFU/g ($p < 0.05$). Similar results were obatined by the TVC counts. At this time the pH of the
246 unspoiled bacons remained quite unchanged, conversely in the spoiled was reduced at level of 5.1
247 ± 0.2. In these samples the greening colour changed to dark green and the slime persisted.
248 These data demonstrated that in the unspoiled bacons no spoilage was observed because LAB did
249 not growth.
250
251 3.2 Microbial identification
252 All the 60 LAB isolates were Gram positive and catalase negative. The isolates were also subjected
253 to molecular identification, and only one species was found: *Leuc. mesenteroides* (Accession
254 Number KC545920.1**)**. The direct identification of the bacteria by a culture independent method
255 (PCR-DGGE and sequencing of the DGGE band) confirmed the traditional identification of the
256 isolates. The DGGE profile of the sample treated using the culture independent method was

257 composed by one only band on the gel, which corresponded to *Leuc. mesenteroides*. Leuconostocs
258 and *L. sakei* are considered to be the bacteria responsible for the spoilage of the cooked meat
259 product stored at 4 °C (Samelis et al., 2000, Metaxopoulos et al., 2002; Cantoni et al., 2008;
260 Vasilopoulos et al., 2010; Comi et al., 2012; Comi and Iacumin, 2012). In this study, the spoiled
261 bacon was contaminated with up to 8 log CFU/g LAB, almost 4 log CFU/g more than the unspoiled
262 cooked bacon ($p < 0.05$). The levels of contamination in spoiled and unspoiled cooked bacon were
263 similar to those present in vacuum and Modified Atmosphere Packaged (MAP) cooked meats from
264 other countries (Nielsen et al., 1983; Samelis et al., 2000). Samelis et al., (2000) found a
265 concentration of LAB up to 7 log CFU/g in sliced cooked ham packaged under vacuum after 15-30
266 days of storage at 4 °C. In our study, *Leuc. mesenteroides* was the only species present in the
267 unspoiled cooked bacon at a concentration level of 4 log CFU/g. Our data are not in agreement with
268 the ones of other authors (Bjorkroth et al., 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008;
269 Audenaert et al., 2010; Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012), that found
270 different LAB. Problably *Leuc. mesenteroides* was the predominant species that was selected in that
271 plant and consequently was the main responsible of the spoilage. However, *Leuconostocs* were
272 highly concentrated in the spoiled cooked bacon. Our data demonstrate that *Leuconostocs* were
273 more profuse because these bacteria grew faster than other LAB at 4 °C, as demonstrated by Comi
274 and Iacumin (2012). It is well-known that in cooked meat products packaged under vacuum,
275 *Leuconostocs*, as well as some others LAB species, are the most prevalent because of the combined
276 effects of pH, Aw (water activity), salinity of the brine and smoke (Samelis et al., 2000; Comi and
277 Iacumin, 2012). During the spoilage, *Leuconostocs* produce lactic acid, acetic acid, ethanol, CO_2
278 and various other compounds that can lead to off-flavours (Cantoni et al., 2008). *Leuc.*
279 *mesenteroides* produced hydrogen peroxide in vitro. This compound is responsible for the greening
280 in meat products because hydrogen peroxide oxidises myoglobin to cholemyoglobin (Collins et al.,
281 1993; Comi and Iacumin, 2012; Jääskeläinen et al., 2013). In the spoiled bacon samples considered
282 in the present study, greening areas were observed by visual analysis.

283 Three main genotipic groups were observed after the identification of the *Leuc. mesenteroides* at
284 strain level. One group isolated from unspoiled bacon had low fermentation speed and growth at 4
285 °C. Conversely one group had high and one medium fermentation and growth speed. The group
286 with higher fermentation and growth speed was isolated from the spoiled bacon. The group with a
287 medium fermentation and growth speed belonged to either unspoiled or spoiled bacon (data not
288 shown). It was assumed that the *Leuconostoc* strain of the spoiled bacon had a greater vitality than
289 the unspoiled one, consequently they were able to produce the spoilage within 90 days of storage. It
290 was assumed that the strains of the unspoiled bacon were less effective and consequently were not
291 able to spoil the bacon within the end of their shelf-life. The lower activity was demonstrated by the
292 concentration reached at 90 days that did not change in respect to 30 days in unspoiled bacon.
293 *Listeria monocytogenes* spp. and *Salmonella* spp. were not detected in any of the tested samples,
294 Clostridia were not detected (less than 10 CFU/g).
295
296 3.3 Analysis of the volatile compounds
297 The analyses of the volatile fractions from the spoiled and unspoiled cooked bacon packaged under
298 vacuum are presented in Table 2. This table shows the mean retention times, the individual
299 compounds and the concentrations of these compounds expressed in µg/kg product from ten
300 analytical runs. The data suggest that the differences observed between the levels of volatile
301 compounds in the spoiled and unspoiled bacon were due to *Leuconostoc* activity. In particular, we
302 found an abundance of heterolactic fermentation products, such as acetic acid, ethanol other
303 carboxylic acids and ketones; aldehydes were not identified because these compounds were most
304 likely transformed into carboxylic acids. Alcohols were mainly present in the spoiled cooked bacon.
305 The analysis was performed on both spoiled and unspoiled cooked bacon after 30 days of storage,
306 because the greening and the slime presence in spoiled appeared after 30 days of storage. To better
307 interpret the results obtained from the analysis of the headspace, the 23 identified substances were
308 split into 5 classes: ketones (4), carboxylic acids (5), alcohols (9), terpenes (2), and miscellanea (3).

309 According to our data, *Leuc. mesenteroides* produce various compounds by fermenting sugars and
310 metabolised amino acids. The concentrations of some volatile compounds were higher in the
311 spoiled bacon than in the unspoiled bacons. Several of the compounds were ascribed to the glucose
312 and amino acid metabolism of the LAB, to the oxidation and auto-oxidation of lipids (Montel et al.,
313 1998) and to the endogenous reactions that occur during cooking (Mottram, 1998). The five
314 identified acids are all typical metabolites from LAB, coliforms and *E.coli* fermentation of sugars
315 and the degradation of amino acids (Leroy et al., 2009). The presence of certain organic acids,
316 including acetic, propanoic, 2-Methyl-propanoic, 3-Methyl-butanoic and Hexanoic acid, is due to
317 the degrading activity of leuconostoc (Comi and Iacumin, 2012; Diez et al., 2009). *Leuc.*
318 *mesenteroides* can increase the concentration of acetic acid and aldehydes in meat product and in
319 blood sausage (Diez et al., 2009). Our data confirm the increasing of the acetic acid concentration
320 but do not show the presence of aldehydes. It could be concluded that the aldehydes were not
321 present because these compounds were most likely transformed into carboxylic acids by *Leuc.*
322 *mesenteroides*, such as hexanal trasformed in hexanoic acid. It is also possible that the acids with
323 more than three carbon atoms can be derived by the lipolytic enzymes in the leuconostocs and in the
324 meat or by lipid oxidation (Chiesa et al., 2006; Comi et al., 2000). Because the brine injected into
325 the meat before production contained up to 0.5% sugars (w/v), we assumed that the organic acids
326 found in the bacon were produced by LAB fermentation. Ethanol was the main alcohol produced
327 and was mainly present in spoiled product. The total concentration of alcohol in the spoiled product
328 was higher than that in the unspoiled product. Leuconostocs most likely produced fewer ketones
329 and carboxylic acids than alcohols. Alcohols can be derived from sugar fermentation (Kandler,
330 1983; Diez et al., 2009), from aldheydes, ketones or from amino acid (leucine, valine and
331 phenylalanine) catabolism and all these activities are typical of *W. viridescens* and leuconostocs
332 (Comi et al., 2014a,b,2011; Deetae et al., 2007; Smit et al., 2005; Bedaguè *et al.,* 1993). In
333 particular, the higher alcohol levels found in the spoiled bacon were produced by the *Leuc.*
334 *mesenteroides* conversion of aldehydes, ketones and amino acids. In addition, the higher alcohol

amounts can be also demonstrated by the higher level of *Leuc. mesenteroides* concentration found in spoiled products, considering that the strains isolated from them had a growth and fermentation speed faster than the strains isolated in unspoiled product. The increase of alcohol in spoiled bacon is associated with the increase of the leuconostoc concentrations (heterofermentative microorganisms). In meat products, the aldehyde levels usually increase with the fermentative activity of a starter consisting of LAB and Coagulase Negative Catalase Positive Cocci or after degradation through the Strecker reaction (Comi et al., 2000). Unlike the results obtained by other authors (Comi et al., 2000; Tjener et al., 2003), no aldehydes were identified in our study. This result can be explained by the degradation and reduction of these compounds by leuconostocs, which converted them in alcohols (Bedarguè et al., 1993; Comi and Iacumin, 2012; Comi et al., 2014a,b).

The identified ketones were primarily derived from the oxidation of fatty acids (2-propanone, 2-butanone, 3-hydroxy-2-butanone, 2-pentanone) and by LAB activities (Berdague et al.,1993; Jääskeläinen et al., 2013). Unlike the ketones identified in other studies (Comi et al., 2000; Tjener et al., 2003, Comi and Iacumin, 2012), diacetyl (2,3-butandione) was not found in our study. This was probably due on the absence of oxygen because the cooked bacon was under vacuum packaged. Jääskeläinen et al., (2013) found that in *Leuc. gasicomitatum* the diacetyl production is minimal in meat packaged under anaerobic condition and on the contrary the respiration enormously increased the accumulation of acetoin and diacetyl under a high-oxygen modified atmosphere. It is also possible that diacetyl may have been reduced to 3-hydroxy-butanone (acetoin), which was found in our study. The absence of diacetyl did not allow to impart a buttery aroma and flavor to spoiled cooked bacon. However, considering the microbial population identified in spoiled cooked hams, it is also possible that the ketones were derived from the degradation of alkanes (Montel et al.,1998). Three out of 4 ketones detected were found in higher concentration in unspoiled than in spoiled. This was explained by a greater activity of the leuconostocs in spoiled bacon that consequently produced higher amount of alcohol and less ketones (Bedarguè et al., 1993; Comi et al., 2014a,b).

Among the aromatic hydrocarbons (terpenes), two compounds were identified both in the unspoiled and spoiled cooked bacon. These compounds are typically found in raw materials and most likely originated from various contaminations in the animal feedstuffs and spices (nutmeg, black pepper) because these compounds can be found in plants (Van Straten, 1977; Comi et al., 2000) that are eaten by animals. For these reasons, a large difference was not observed between the terpene concentrations of the spoiled and unspoiled cooked bacon.

3.4 Challenge test

Table 3 shows the results obtained using the biopotective culture: their use can eliminate the spoilage produced by leuconostocs. In fact, cooked bacon with bio-protective cultures did not present greening, slime or inflated packaging at the end of the shelf life. In contrast, a sticky-white slime and a greening colour were observed in the cooked bacon inoculated only with *Leuc. mesenteroides*. The bio-protective cultures grew during the storage of the products, and at the end of the shelf-life, the ratio *Leuc. mesenteroides/ Lc. lactis* subsp. *lactis* was about 1/100. In fact the DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a sample decimal dilution of 10^{-3}, whereas DNA of *Lc. lactis* subsp. *lactis* at a decimal dilution of 10^{-5} (Table 4). On the other hand, at the end of the shelf-life, the ratio of *Leuc. mesenteroides/L. sakei,* was about 1/1000. In fact the DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a decimal dilution of 10^{-3}, whereas the DNA of *L. sakei* at a dilution level of 10^{-6} (Table 4). On the basis of the obtained results, can be assumed that the bioprotective coltures, competing for the substrate with *Leuc. mesenteroides*, inhibited their growth. This assumption is also confirmed by the results obtained for the control sample (Lot 1), where the DNA of *Leuc. mesenteroides* was identified at a dilution level of 10^{-6} (Table 4). *Leuc. mesenteroides* concentration in Lot 1 was 6.0 ± 0.3 UFC/g.

Consequently, the growth of the bio-protective cultures eliminated the spoilage caused by leuconostocs, and the products resulted acceptable at the end of the shelf life (90 days).

386 The use of LAB as bio-protective cultures to prolong the shelf life of the meat products, including
387 cooked meat product, is a new concept that has been suggested by many authors (Metaxopoulos et
388 al.,2002; Vermeiren et al.,2004; Comi et al.,2011). Kotzekidou and Bloukas (1996) noticed that
389 cooked ham supplemented with protective cultures had lower total aerobic bacteria, micrococci,
390 staphylococci and *B. thermosphacta* counts than control hams, which had higher populations of
391 LAB and lower populations of pseudomonads. Additionally, Metaxopoulos et al., (2002) and
392 Vermeiren et al., (2006) concluded that LAB affected the spoilage microflora growth and did not
393 negatively affect the organoleptic properties of the products. Comi et al., (2012) demonstrated that
394 starter cultures (*L. sakei*, *L. curvatus* and *Lc. lactis* subsp. *lactis*) extend the shelf life of cooked ham
395 slices packed in MAP. The microorganisms influenced the flavour, odour and stability of the colour
396 of the sliced cooked ham.
397
398 3.5 Sensorial analysis
399 The acceptability of the bacon added with starter cultures was confirmed with the triangular test by
400 the panel composed by 20 not-professional assessors. They established that did not exist any
401 difference between Lot A and B (with bio-protective culture): the slice resulted compact and
402 homogeneous; the lean part was a ruby red color and the color of the fat was white, typical of the
403 product; the consistency was compact but not elastic; the bouquet was delicate and distinctive; taste
404 was sweet and delicate and there was no perception of spices or flavors; acidity was not perceived.
405 On the other hand, Lot C was indicated as different by the 100% of the assessors, who stressed as
406 follows: the slice resulted non compact and the presence of irregular holes was observed; the lean
407 part was a ruby red color with the presence of green coloring and slime; the color of the fat was
408 white, typical of the product; the consistency was compact but not uniform; the bouquet was altered
409 and not distinctive of the product.
410
411 **4. Conclusion**

Data demonstrated that bio-protective cultures inhibited *Leuc. mesenteroides* growth and eliminated the greening colour of the meat, the slime, the package inflation, the off-flavours and the off-odours. Therefore, the bio-protective cultures evaluated in this study can improve the shelf life and eliminate the growth of spoilage microorganisms. In particular this is the fisrt time that a *Lc. lactis* subsp. *lactis* is used in order to improve the shelf-life of cooked meat as cooked bacon.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1: Process steps of traditional cooked bacon production

Step	Temperature/time
Raw material	4 ± 2 °C
Trimming, Squaring	12 °C
Brine injection	7 °C
Churning	7 °C – 8 hours
Resting	2-4 °C – 7 days
Drying	44-55 °C – 1 hour
Smoking	66 °C – 24 hours
Cooking	72 – 78 °C
Chilling	2-4 °C
Packaging	4 °C
Pasteurization	85 °C – 15 minutes
Storing	4 ± 2 °C

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526 **Table 2**: Volatile compounds in unspoiled and spoiled bacon

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RT	Compounds	Unspoiled		Spoiled	
		Mean	(±) SD	Mean	(±) SD
	Ketones				
2.29	2-Propanone	2.51	0.02a	1.65	0.08b
3.17	2-Butanone	6.04	0.03b	9.33	0.01a
4.72	2-Bentanone	8.45	0.07a	0.28	0.01b
17.63	3-Hydroxy-2-butanone	37.35	0.64a	5.80	0.19b
Sum		**54.35**		**17.06**	
	Alcohols				
3.87	Ethanol	16.90	0.08b	26.11	0.02a
6.64	2-Butanol	1.28	0.04b	5.82	0.26a
7.28	1-Propanol	4.54	0.12b	28.18	0.71a
10.22	2-Methyl-1-propanol	0.75	0.30b	2.73	0.26a
11.1	2-Propen-1-ol	0.04	0.01b	0.30	0.03a
11.6	2-Pentanol	0.17	0.02b	0.63	0.11a
15.42	3-Methyl-1-butanol	4.83	0.14b	8.36	0.02a
16.76	3-Methyl-3-buten-1-ol	3.07	0.04a	0.15	0.02b
25.17	2.3-Butanediol	3.18	0.04a	0.12	0.01b
Sum		**28.76**		**72.39**	
	Terpenes				
9.67	α-Pinene	0.24	0.01a	0.28	0.12a
12.06	δ-3-Carene	0.04	0.02a	0.17	0.03a
Sum		**0.28**		**0.45**	
	Carboxylic acid				
22.23	Acetic acid	5.48	0.11b	7.51	0.21a
24.28	Propanoic acid	0.49	0.10b	0.17	0.02a
24.95	2-Methyl-propanoic acid	0.18	0.00a	0.08	0.00b
27.04	3-Methyl-butanoic acid	1.88	0.07a	1.04	0.02b
30.34	Hexanoic acid	0.09	0.04b	0.16	0.02a
Sum		**8.11**		**8.97**	
	Miscellany				
3.05	Acetic acid ethyl-ester	2.26	0.04a	0.88	0.07b
25.98	Butirolactone	0.14	0.03a	0.12	0.01a
31.12	Dimethylsulfone	0.02	0.01a	0.03	0.01a
Sum		**2.42**		**1.02**	

Legend: Mean (mean of 10 samples analysed in triplicate) expressed in µg/kg; Sum of compounds; RT: Retention time. SD: Standard deviation. Data represent the means ± standard deviations of all the samples; Mean with the same letters within a row (following the values) are not significantly differently ($P < 0.05$)

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533 **Table 3**: Physico-chemical results of the bio-protective effect of the LAB starter used,
534 versus *Leuconostoc mesenteroides*
535

Lot	Microorganisms	Greening	Slime	pH
1	*Leuc. mesenteroides*	+	+	5.3 ± 0.2* a
2	*Leuc. mesenteroides vs Lc. lactis*	-	-	5.4 ± 0.1* a
3	*Leuc. mesenteroides vs L. sakei*	-	-	5.4 ± 0.2* a

Legend: Mean (mean of 10 samples analysed in triplicate) expressed in µg/kg; Sum of compounds; RT: Retention time. SD: Standard deviation. Data represent the means ± standard deviations of all the samples; Mean with the same letters within a row (following the values) are not significantly differently ($P < 0.05$)

536 **Table 4:** Plate dilution at which the identified species were detected

Lot	Microorganism inoculated	Serial decimal dilution				
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	*Leuc. mesenteroides*	+	+	+	+	-
2	*Leuc. mesenteroides*	+	-	-	-	-
	Lc. lactis	+	+	+	-	-
3	*Leuc. mesenteroides*	+	-	-	-	-
	L. sakei	+	+	+	+	-

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